Lung endothelial cell proliferation with decreased shear stress is mediated by reactive oxygen species

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Milovanova, Tatyana, Shampa Chatterjee, Yefim Manevich, Irina Kotelnikova, Kris DeBolt, Muniswamy Madesh, Jonni S. Moore, and Aron B. Fisher. Lung endothelial cell proliferation with decreased shear stress is mediated by reactive oxygen species. Am J Physiol Cell Physiol 290:C66–C76, 2005. First published August 17, 2005; doi:10.1152/ajpcell.00094.2005.—Acute cessation of flow (ischemia) to pulmonary endothelial cells (ECs) either in situ or after flow adaptation in vitro increased production of reactive oxygen species (ROS) from NADPH oxidase. We postulated that ROS are a signal for initiating EC proliferation associated with the loss of shear stress. Flow cytometry was used to identify proliferating CD31-positive pulmonary microvascular endothelial cells (mPMVECs) from wild-type, Kir6.2−/−, and gp91phox−/− mice. mPMVECs were labeled with PKH26 and cultured in artificial capillaries for 72 h at 5 dyn/cm2 (flow adaptation), followed by 24 h of stop flow or continued flow. ROS production during the first hour of ischemia was markedly diminished compared with wild-type mice in both types of gene-targeted mPMVECs. Cell proliferation was defined as the proliferation index (PI). After 72 h of flow, >98% of PKH26-labeled wild-type mPMVECs were at a single peak (PI 1.0) and the proportion of cells in the S+G2/M phases were at 5.8% on the basis of cell cycle analysis. With ischemia (24 h, PI increased to 2.5 and the ratio of cells in S+G2/M phases were at 35%. Catalase, diphenyleneiodonium, and cromakalim markedly inhibited ROS production and cell proliferation in flow-adapted wild-type mPMVECs. Significant effects of ischemia were not observed in Kir6.2−/− and gp91phox−/− cells. ANG II activation of NADPH oxidase was unaffected by KATP gene deletion. Thus loss of shear stress in flow-adapted mPMVECs resulted in cell division associated with ROS generation by NADPH oxidase. This effect requires a functioning cell membrane KATP channel.

WE HAVE SHOWN PREVIOUSLY that abrupt reduction in shear stress (ischemia) to pulmonary endothelial cells (ECs) either in situ or after flow adaptation in vitro increased production of reactive oxygen species (ROS), activation of the transcription factors NF-κB and activator protein-1 (AP-1), and cellular proliferation (4, 13, 15, 18, 24). These effects appear to be part of a signaling cascade associated with altered mechanical stress. Studies with the intact lung indicated that depolarization of the EC plasma membrane represents an initial event with altered mechanotransduction and precedes ROS generation (18). EC membrane depolarization and ROS generation with flow cessation in both the intact lung and flow-adapted ECs were prevented by pretreatment with the KATP channel agonist cromakalim and were mimicked by treating lungs with glibenclamide (glyburide), a KATP blocker (3, 18, 25). We have proposed that KATP channels in lung ECs are responsible for maintaining membrane potential with normal shear stress and that channel inactivation by loss of shear leads to membrane depolarization (9). Ischemia-mediated cell membrane depolarization and ROS generation also have been demonstrated for the aortic endothelium (14).

The source of ROS after membrane depolarization appeared to be the plasma membrane NADPH oxidase, because the response was not present in lungs from gp91phox gene-targeted mice and was inhibited by diphenyleneiodonium (DPI), an inhibitor of flavoproteins (4). EC express NADPH oxidase subunits that are identical to those found in phagocytes. These include the integral membrane protein flavocytochrome b558, composed of gp91phox (also known as Nox2) and p22phox subunits, and the cytosolic components p40phox, p47phox, and p67phox (8). Additional NADPH oxidase-associated flavoproteins (e.g., Nox4) also are present in ECs (8).

Ischemia also was associated with increased EC division (15, 24). Flow-adapted bovine and rat pulmonary artery ECs demonstrated twice as much [3H]thymidine incorporation after interruption of medium flow while maintaining oxygenation of ECs that had been preadapted to shear stress in culture. Through the use of inhibitors and ECs from gene-targeted mice, we show the primary role of KATP channels and NADPH oxidase in generating the signals that result in EC proliferation with altered shear stress.

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METHODS

EC isolation. C57BL/6 (wild type) and gp91phox−/− (stock no. 2365) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Kir6.2−/− were originally a gift from Dr. S. Seino (Chiba University, Chiba, Japan) and were bred in our facilities. The gp91phox−/− and Kir6.2−/− mice had been backcrossed to the C57BL/6 background for >10 and 5 generations, respectively. Animal protocols were approved by the University of Pennsylvania Animal Care and Use Committee.

Mouse pulmonary microvascular endothelial cells (mPMVECs) were isolated from the lungs of wild-type and gene-targeted mice as described elsewhere (10, 27). Briefly, freshly harvested mouse lungs were treated with collagenase, followed by isolation of cells by adherence to magnetic beads coated with MAb to platelet endothelial cell adhesion molecules (PECAMs) obtained from BD Biosciences Pharmingen (Palo Alto, CA). mPMVECs were propagated in DMEM supplemented with 10% FBS, nonessential amino acids, and penicillin/streptomycin. Cells were maintained under static culture conditions for several passages before being subjected to flow. The endothelial phenotype of the preparation was routinely confirmed by evaluating cellular uptake of 1,1'-dioctadecyl-3,3',3',3'-tetramethylethylene-dioctocarbocyanine perchlorate (DiI)-acetylated LDL (DiI-AcLDL) and immunostaining for PECAM. As a negative control for immunofluorescence, A549 cells, a human epithelial cell line derived from a bronchoalveolar carcinoma, were obtained from the American Type Culture Collection (Manassas, VA).

The presence of the Kir6.2 and gp91phox proteins in ECs was evaluated by immunofluorescence, immunoblot, and flow cytometry using a Kir6.2 PAb (Alomone Laboratories, Jerusalem, Israel) or a gp91phox MAb (BD Biosciences Pharmingen). For immunoblot analysis, whole cell lysates were analyzed for Kir6.2 and plasma membranes were isolated using sucrose density gradient centrifugation (11) and analyzed for gp91phox. Immunoblotting was performed using the two-color Odyssey Li-Cor (Lincoln, NE) Western blot analysis technique (21) according to previously described methods for sample preparation (24). Secondary dual-labeled antibodies were IrDye 800 goat anti-rabbit (Rockland, Gibertsville, PA) for the green 800-nm channel and Cy5.5 or Alexa 680 goat anti-mouse (Molecular Probes, Eugene, OR) for the red 700-nm channel. After we performed SDS-PAGE and transfer, we scanned the membrane with the Odyssey two-color scanner and protein content was quantitated with Odyssey software on the basis of fluorescence.

Cell culture and simulated ischemia. ECs between passages 5 and 15 were cultured under a flow of culture medium (DMEM) using commercially available artificial capillary technology (FiberCell Systems, Frederick, MD) as described previously (9, 15, 23, 24). Before cell seeding, the inner lumen of the “capillary” fibers was coated with ProNectin F (Protein Polymers, San Diego, CA). Freshly harvested cells from two confluent T-75 flasks containing ECs (14–16 × 10⁶ cells) were seeded into each cartridge. The cartridge environment was maintained at 37°C and 5% CO₂ in a humidified incubator. To allow attachment of ECs to the capillary fibers, the perfusing medium was routed to the abluminal side for a 24-h period. The perfusion circuit then was rerouted to the luminal side so that cells were subjected to shear. The nominal times described in this report do not include the initial 24-h attachment period used for all experiments. Cells were cultured under steady laminar flow, generally for 48–72 h, using a flow rate sufficient to generate 5 dyn/cm² shear stress. Shear rate was calculated from specifications supplied by the manufacturer of the FiberCell modules.

Ischemia was simulated by rerouting the flow from the luminal to the abluminal compartment. This protocol eliminated shear stress but allowed continued cellular oxygenation and access to nutrients. Using an oxygen electrode and medium samples obtained from the cartridge lumen, we have shown previously that PO₂ during abluminal flow (ischemia) is similar to that of control (24). Experimental cells were grown under flow for 72 h, followed by a 1-h stop flow (ischemia) for measurement of ROS or 24-h stop flow for assessment of cell proliferation. The corresponding control cells were grown under flow or static conditions for either 73 or 96 h. Inhibitors or other agents were added during the final 1 h of flow adaptation and remained present during the subsequent 1- or 24-h ischemia or continued flow periods. At the end of the control (static or flow) or experimental (ischemia) period, cells were removed from the cartridges by treatment in the incubator with 0.25% trypsin for 5 min or Accutase enzymes (Chemicon International, Temecula, CA) at 10 ml/carteHD for 10 min, centrifuged at 1,400 g at 4°C for 5 min, and then analyzed using flow cytometry or confocal microscopy.

In additional experiments, cells grown under flow conditions for 72 h in the cartridges were trypsinized, aliquoted at 2.5 × 10⁶ per well into six-well plates, and cultured in the incubator at 37°C and 5% CO₂ for 24 and 48 h. Cells were observed by phase-contrast microscopy and then trypsinized from the wells to determine the number of cells by counting them with a hemocytometer.

Flow cytometry. Flow cytometry was performed with a four-color dual-laser FACSCalibur device (Becton Dickinson, San Jose, CA) as described previously (15). Monoclonal antibody to CD31 (BD Biosciences Pharmingen) conjugated with FITC (Caltag, Burlingame, CA) or streptavidin-allophycocyanin (BD Biosciences Pharmingen) and uptake of DiI-Ac-LDL were used to identify ECs.

Cell proliferation. Cell proliferation was studied as described previously (15). Cells were labeled with the lipophilic dye PKH26 under conditions that were previously established to stain cells homogeneously with little loss of viability and at appropriate levels for spectral compensation (15). After being labeled, cells were seeded into cartridges for culture under static or flow conditions and used for ischemia experiments. For analysis of cell proliferation by flow cytometry, a gate was set around the CD31-positive cells to identify the EC population (15). The proliferation index (PI) was calculated using the ModFit program as the proportion of cells that had divided (15).

Cell cycle phases of the diploid population were analyzed by performing flow cytometry using the ModFit program on the basis of cellular DNA content (15). For the terminal TdT-mediated dUTP nick-end labeling (TUNEL) assay performed to detect apoptosis, we used the APO-Direct kit (BD Biosciences Pharmingen) (15). Perfixation apoptotic cells included in the kit and also EC treated for 24 h with 1.2 mg/ml TNF-α (BD Biosciences Pharmingen) were used as positive controls. Cells incubated with the staining solution in the absence of terminal deoxynucleotidyl transferase enzyme served as a negative control.

ROS generation. ROS generation was assessed by preloading cells with 10 μM 2',7'-dichlorofluorescein (H₂DCF) diacetate (Kodak, Rochester, NY) and measuring its conversion to fluorescent DCF (15). Cells were flow adapted for 72 h and then subjected to 1-h ischemia. H₂DCF diacetate was added to the cell perfusate during the final 1 h of flow adaptation. In some experiments, 5,000 U/ml bovine liver catalase (Calbiochem, La Jolla, CA), 3,000 U/ml bovine liver SOD (Calbiochem), 10 μM N⁴-nitro-l-arginine methyl ester (l-NAME; Sigma, St. Louis, MO), 10 μM DIP (ICN Biochem), or 30 μM n-cromakalim (Sigma) was added along with H₂DCF. The perfusate during the dye-loading and subsequent experimental periods for ROS measurement was Krebs-Ringer bicarbonate (KRB) solution, pH 7.4. In additional experiments, ANG II (2 μM) or glyburide (10 μM) was added at the end of the 72-h flow adaptation period and luminal flow was continued for an additional 1 h. These latter cells were not ischemic. At the end of the experimental period, cells were trypsinized from the cartridges and analyzed by performing flow cytometry or confocal microscopy (Radiance 2000; Bio-Rad Laboratories, Hercules, CA). For the latter, cells were centrifuged at 1,400 g for 5 min and then placed on a glass slide for observation at ×600 magnification.
Statistics. Results are expressed as means ± SE for three or more independent experiments. Significance was determined using ANOVA or Student’s t-test as appropriate with SigmaStat software (Jandel Scientific, San Jose, CA), and the level of statistical significance was defined as \( P < 0.05 \).

RESULTS

mPMVEC phenotype and growth characteristics. In the present study, we used three cell lines of mPMVECs generated in our laboratory: wild-type, Kir6.2\(^{−/−}\), and gp91\(\text{phox}^{−/−}\). Endothelial phenotypic identity for these cell lines was confirmed using CD31 FITC and Dil-Ac-LDL staining (Fig. 1). All cells in the population were positive for these two markers. A549 cells were used as a negative control (data not shown). As expected, immunofluorescence and immunoblot analysis failed to detect Kir6.2 in the Kir6.2\(^{−/−}\) cells or gp91\(\text{phox}^{−/−}\) in the gp91\(\text{phox}^{−/−}\) cells; the expression level of Kir6.2 in the gp91\(\text{phox}^{−/−}\) cells and of gp91\(\text{phox}\) in the Kir6.2\(^{−/−}\) cells were similar to the expression level observed in the wild-type cells (Fig. 2). The molecular mass for gp91\(\text{phox}\) shown in Fig. 2 is consistent with that of the nonglycosylated protein (22). Wild-type mPMVECs seeded at subconfluent density and cultured under static conditions showed a doubling time of 24–36 h and a PI of 8–10 at 24 h that were not altered by the presence of DPI or cromakalim (data not shown). Subconfluent statically cultured cells from gp91\(\text{phox}^{−/−}\) mice showed proliferation levels similar to those of wild-type mice, while cells from Kir6.2\(^{−/−}\) mice showed modestly increased proliferation (data not shown).

ROS generation with ischemia. Oxidation of H\(_2\text{DCF}\) as determined using confocal microscopy was barely detectable in wild-type, statically cultured mPMVECs (data not shown) and in flow-adapted mPMVECs measured during a final hour of continued flow (Fig. 3). DCF fluorescence in wild-type mPMVECs was markedly increased with 1-h ischemia (Fig. 3b). Increased fluorescence was observed in nearly all of the cells examined. The increase in DCF fluorescence with ischemia was abolished by catalase (Fig. 3c) but was not significantly inhibited by SOD (Fig. 3d) or L-NAME (Fig. 3e). This suggests that H\(_2\text{O}_2\), presumably generated by dismutation of O\(_2^-\), is the major oxidant for H\(_2\text{DCF}\) in this system.

Fig. 1. Anti-CD31 (platelet endothelial cell adhesion molecules, PECAMs) FITC and 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (Dil)-acetylated LDL (Dil-Ac-LDL) phenotyping of pulmonary microvascular endothelial cells (mPMVECs) using confocal microscopy (A) and flow cytometry (B). A: endothelial cells (ECs) demonstrating anti-CD31 fluorescence (a–c) and Dil-Ac-LDL fluorescence (d–f), and phase-contrast microscopy (g–i) of wild-type ECs (a, d, and g), Kir6.2\(^{−/−}\) ECs (b, e, and h) and gp91\(\text{phox}^{−/−}\) ECs (c, f, and i). B: histograms of anti-CD31 FITC and Dil-Ac-LDL-stained cells. Lower-cased letters refer to the same conditions shown in A. Cells stained with isotype FITC (ctr) were used as a negative control. au, arbitrary units.
generation with ischemia also was inhibited by the presence of the flavoprotein inhibitor DPI (Fig. 3Af), or the KATP channel agonist cromakalim (Fig. 3Ag). DPI inhibits NADPH oxidase by interaction with its flavoprotein component (gp91phox), while cromakalim activates KATP channels and prevents the ischemia-mediated membrane depolarization that results in NADPH oxidase activation (6, 9, 18). H2DCF oxidation with ischemia was markedly decreased in Kir6.2/H11002/H11002 mPMVECs (Fig. 3Ah) and was absent in gp91phox/H11002/H11002 mPMVECs (Fig. 3Ai). The results for changes of DCF fluorescence evaluated using microscopy were confirmed by performing flow cytometry (Fig. 3B).

Cellular proliferation with ischemia. Cell proliferation was determined by analyzing cells that had been labeled with PKH26 before seeding. Cells were seeded at a density that promoted confluence as determined previously by cell cycle analysis and immunostaining with PCNA (15). The PI of cells at day 0 was set to 1.0. The fluorescence distribution for cells cultured for 96 h under flow for wild-type mice as well as cells from Kir6.2/H11002/H11002 and gp91phox/H11002/H11002 mice showed a single peak, with a PI unchanged from day 0 (Fig. 4, Aa, Ae, and Ag). Significant proliferation (PI 2.6) was observed for wild-type mPMVECs that had been flow adapted for 72 h followed by 24-h ischemia (Fig. 4Ab). Cell proliferation was essentially unaffected by the presence of SOD (PI 2.4) but was significantly inhibited by the presence of catalase (PI 1.2) (data not shown). Both cromakalim and DPI also significantly inhibited proliferation of flow-adapted cells that were then subjected to ischemia (Fig. 4, Ac and Ad). These results are similar to our previously reported results for ischemia of rat pulmonary microvascular ECs (15). Compared with ECs from wild-type mice, proliferation with ischemia was markedly diminished in ECs from Kir6.2/H11002/H11002 and gp91phox/H11002/H11002 mice (Fig. 4, Af and Ah). The PI results for three experiments under each condition are shown in Fig. 4B.

The number of cells that could be trypsinized from the cartridges after 96 h of culture was evaluated for the three EC lines (wild type, Kir6.2/H11002/H11002, gp91phox/H11002/H11002). Studies using wild-type cells cultured under static conditions indicated that the confluent condition was a yield of 17–18 × 10⁶ cells/cartridge.
For cells cultured under flow conditions, the yield was $10^6$ cells for all three cell types, indicating near confluence (Table 1). With cessation of flow, the yield of wild-type cells increased to the confluent cell level, while the yield of gp91phox cells was only slightly greater than the number for culture under flow conditions and the yield of Kir6.2 cells was intermediate between the static and continuous flow conditions (Table 1). These results are compatible with the flow cytometric data indicating that the absence of KATP channels or NADPH oxidase inhibits the proliferative response to altered shear stress.

The proliferative response to loss of shear stress also was evaluated by performing another type of experiment. At the end of the flow adaptation period (72 h), cells were removed from the cartridges and plated in 6-well plates for culture under static conditions. Agents (10 μM DPI or 30 μM cromakalim) were added during the final hour of flow adaptation in the cartridges and the subsequent 24-h static culture period in the six-well plates but were then removed during the 24- to 48-h culture period. At 24 h of static culture, wild-type ECs had increased by 233% above the starting number (Fig. 5). With DPI treatment, the mean increase was only 33% within 24 h and only 47% with cromakalim treatment (Fig. 5). Proliferation also was much less compared with wild-type mice in gene-targeted cells, with increases of only 20% and 7%, respectively, in Kir6.2−/− and gp91phox−/− ECs (Fig. 5). Cells in all conditions showed proliferation during the subsequent 24- to 48-h culture period (Fig. 5). The number of cells per well at 48 h was not significantly different from that observed in wild-type mice for cromakalim treatment and Kir6.2−/− cells
and only slightly less than that found in wild-type cells for DPI treatment and gp91phox−/− cells (Fig. 5). Thus wild-type cells postinhibitor, as well as gene-targeted cells, were proliferation competent and showed a rate of proliferation during the 24- to 48-h period that was similar to or greater than that of control cells.

Cell cycle analysis with ischemia. mPMVECs cultured under static conditions revealed ~50% of cells in S+G2/M...
phases for all three cell lines (wild type, Kir6.2−/−, and gp91phox−/−) (Fig. 6, Ae, Ab, and Ac). After flow adaptation for 72 h, only 5.8±7.4% of cells from the three cell lines were in S+G2/M phases (Fig. 6, Ad, Ae, and Af). With ischemia for 24 h, wild-type mPMVECs demonstrated a sixfold increase in the number of cells in S+G2/M phases (Fig. 6Ag). With ischemia, the percentage of cells in S+G2/M from Kir6.2−/− mice increased only 2.5-fold compared with continuous flow, whereas the percentage of gp91phox−/− cells in S+G2/M was essentially unchanged (Fig. 6, Ag, Ah, and Ai). The decreased percentage of cells in S+G2/M in the two knockout cell lines compared with wild type was statistically significant (Fig. 6B).

Apoptosis of ECs. TUNEL assay was used to investigate the possibility that apoptosis occurred and that loss of cells was due to the stimulus for cellular proliferation with ischemia. Under culture static conditions, 2–3% of cells were TUNEL positive (data not shown). The percentage of apoptosis was similarly low for mPMVECs from each of the three cell lines that were flow adapted for 96 h or flow adapted for 72 h, followed by 24-h ischemia (Table 2). As a positive control, a population of ~25% TUNEL-positive cells was demonstrated after 24-h incubation with TNF-α (data not shown).

ROS generation with ANG II and glyburide. To examine the correlation between cell membrane depolarization and ROS generation in these cells, we studied the effect of ANG II and glyburide on DCF oxidation. ANG II activates NADPH oxidase through activation of serine/threonine kinases (19) and possibly activation of nonspecific cation or Cl− channels (12), while glyburide (glybenclamide) is a KATP channel antagonist (3). In wild-type cells, both ANG II and glyburide resulted in ROS production as indicated by DCF oxidation (Fig. 7). In gp91phox−/− cells, neither agent elicited ROS production (Fig. 7), indicating that NADPH oxidase is the source of ROS with these treatments in wild-type cells. In Kir6.2−/− cells, ANG II elicited ROS generation but glyburide was ineffective (Fig. 7). This result indicates that ROS generation induced by glyburide but not by ANG II in ECs requires the presence of the KATP channel.

DISCUSSION

We previously have demonstrated ROS production by ECs as a response to abrupt loss of shear stress (ischemia) in both an intact lung model and in vitro culture cell models (Refs. 4, 6, 9, 13, 15, 18, 23, 24, 27). These studies also have shown that simulated ischemia in vitro results in the proliferation of pulmonary ECs (15, 24). This response appeared to be dependent on ROS generation, because it was prevented by both DPI and cromakalim. ROS generation in the altered shear stress models is inhibited by DPI, a flavoprotein blocker that inhibits ROS production by NADPH oxidase, and by cromakalim, a KATP channel agonist that prevents membrane depolarization with ischemia (18). We have postulated that removal of the mechanical stimulus associated with normal perfusion leads to activation of the O2−-generating cell membrane NADPH oxidase and that the link between altered shear stress and NADPH activation is endothelial plasma membrane potential (4). We propose that KATP channels are largely responsible for the resting cell membrane potential and are normally maintained in the open state by shear stress. Cell membrane depolarization results from inactivation of the channels associated with loss of shear (5, 9).

Evidence that NADPH oxidase activation is linked to cell membrane depolarization has been obtained by studying temporal relationships and using inhibitors. Imaging of the ischemic response in isolated rat lung and flow-adapted bovine pulmonary artery endothelial cells indicated that cell membrane depolarization preceded ROS generation (13, 18). Cromakalim, a KATP channel agonist that prevents membrane depolarization with ischemia, also prevented activation of NADPH oxidase (18). Conversely, treatment with glyburide, an agent that inactivates KATP channels, resulted in ROS generation in the endothelium of intact lung (3) as well as in ECs in culture (present study). Furthermore, cell membrane depolarization produced by treatment with high K+ activated endothelial ROS production in rat lungs (3, 4) and isolated cells (2). In the present study, deletion of the KATP channel resulted in marked blunting of ROS generation with ischemia. By contrast, treatment with ANG II, which also activates NADPH oxidase, stimulated ROS production similarly in wild-type and KATP gene-targeted ECs (15). On the other hand, ROS production with stop

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The major goal of the present study was to evaluate the roles of KATP channels and cell membrane NADPH oxidase in the endothelial proliferative response associated with ischemia using mouse models with knockout of gp91phox (NADPH oxidase) and Kir6.2 (KATP Channels). ECs from wild-type mice showed ROS generation with stop flow as indicated by the oxidation of DCF that was inhibited with DPI and cromakalim, confirming our previous results with rat pulmonary microvascular ECs (15). On the other hand, ROS production with stop

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**Table 1. Endothelial cell yield from cartridges**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Wild-Type Cells, 1 × 10⁵</th>
<th>Kir6.2−/− Cells, 1 × 10⁶</th>
<th>gp91phox−/− Cells, 1 × 10⁶</th>
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<tr>
<td>Continuous flow</td>
<td>13.6±2.9</td>
<td>14.0±2.15</td>
<td>14.0±1.6</td>
</tr>
<tr>
<td>Ischemic</td>
<td>18.1±2.1</td>
<td>16.0±0.89†</td>
<td>14.7±2.1†</td>
</tr>
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</table>

Values are means ± SE for the number of cells obtained per cartridge; n = 4 experiment. *P < 0.05 vs. continuous flow; †P < 0.05 vs. corresponding wild type. Cartridges were seeded with 14–16 × 10⁶ cells, which were cultured for 96 h and then released from the cartridges by trypsinization. Cells were cultured under 5 dyn/cm² shear stress for 72 h, followed by an additional 24 h of flow or 24 h of ischemia.

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**Fig. 5. Cell proliferation of flow-adapted mPMVECs after ischemia.** Cells were cultured under 5 dyn/cm² shear stress for 72 h, followed by trypsinization, and then plated in 6-well dishes at 2.5 × 10⁵ cells/well for 24- or 48-h culture under static conditions. At the end of incubation, cells were trypsinized from wells and the number of cells was counted using a hemocytometer. DPI or cromakalim was added during the last hour of flow adaptation and removed after 24-h culture. Dotted line crossing bars along x-axis indicates the number of cells plated at time 0. Values are means ± SE; n = 3 experiments. *P < 0.05 vs. corresponding wild-type ECs with no inhibitors.

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Fig. 6. Flow-cytometric analysis of cell cycle for wild-type, Kir6.2−/−, and gp91phox−/− mPMVECs in response to ischemia. A: mPMVECs were cultured for 72 h under static or flow adaptation conditions, followed by 24 h of continued flow or ischemia. Cells were seeded at $1 \times 10^6$ for static culture and at $18 \times 10^6$ for flow adaptation. Histograms of DNA content of mPMVECs were generated using fluorescence-activated cell sorting after removal of the cells from the cartridges. The distribution of diploid cells in G1/G0, S, and G2/M phases is expressed as a percentage of total cells. A: static mPMVECs at 96 h of culture for wild-type ECs (a), Kir6.2−/− ECs (b), and gp91phox−/− ECs (c). Flow-adapted mPMVECs at 96 h of culture for wild-type ECs (d), Kir6.2−/− ECs (e), and gp91phox−/− ECs (f). Ischemia after 24 after 72 h of flow adaptation for wild-type ECs (g), Kir6.2−/− ECs (h), and gp91phox−/− ECs (i). B: histograms indicating means ± SE for cells in G2/M+S phases as a percentage of total cells; $n = 6$ experiments. *$P < 0.05$ vs. wild type for each condition; # $P < 0.05$ vs. the corresponding flow condition.
flow did not occur with gp91phox−/− ECs and was markedly attenuated in Kir6.2−/− ECs. Because the absence of ROS generation with ischemia occurs through different mechanisms in these two cell types, their use is a strong approach to use in the evaluation of the role of ROS in EC proliferation with ischemia.

To evaluate the proliferation of cells, we used flow cytometry of cells labeled with fluorescent dyes to stain the cytoplasm or the lipid bilayer of the plasma membrane. PKH26 labeling of ECs is a well-described technique that we have used previously to evaluate in vitro proliferation in rat pulmonary microvascular ECs (15). Cell proliferation also was evaluated by counting the number of cells that could be trypsinized from

<table>
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<th>Conditions</th>
<th>Wild Type, %</th>
<th>Kir6.2−/−, %</th>
<th>gp91phox−/−, %</th>
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<tr>
<td>Static culture</td>
<td>2.0 ± 1.1</td>
<td>1.8 ± 0.32</td>
<td>3.0 ± 0.88</td>
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<td>Continuous flow</td>
<td>3.1 ± 1.2</td>
<td>2.0 ± 0.78</td>
<td>3.5 ± 1.1</td>
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<tr>
<td>Ischemic</td>
<td>3.3 ± 1.1</td>
<td>2.4 ± 0.86</td>
<td>3.0 ± 1.1</td>
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Values are means ± SE (n = 4 experiments) for percentage of cells that were positive for TUNEL staining. None of the values are significantly different from the corresponding wild-type value. These assays used the cells described in Table 1. mPMVECs, murine pulmonary microvascular endothelial cells; TUNEL, terminal TdT-mediated dUTP nick-end labeling.

Fig. 7. Effect of ANG II and glyburide on DCF fluorescence analyzed using flow cytometry. A: mPMVECs were flow adapted for 24 h and then analyzed for ROS production as indicated by DCF fluorescence during 1 h of continued flow in the presence of 2 μM ANG II or 10 μM glyburide. Results are shown for mPMVECs from wild-type mice (a–c), Kir6.2−/− mice (d–f), and gp91phox−/− mice (g–i). B: DCF fluorescence for experiments shown in A expressed as means ± SE; n = 4 experiments. *P < 0.05 vs. corresponding wild type.
the cell culture cartridges and by cell cycle analysis of cells stained with propidium iodide. Each of these assays demonstrated that stop flow stimulated proliferation in wild-type ECs. Proliferation occurred even though cells were seeded at a density that promotes confluence, indicating that the stimulus by ROS overcomes contact inhibition as shown by our previous studies (15). Similar results have been reported for bovine aortic endothelial cells, in which proliferation of confluent cells was observed after stimulation with oxidized LDL (16) or glucose oxidase (17) to produce ROS. Evidence that the response in the present experiments is specific and dependent on ROS is inhibition of ischemia-mediated proliferation of flow-adapted cells by DPI and cromakalim; these agents had no effect on the proliferation of cells cultured under static conditions. Studies of cells grown in wells (Fig. 5) show that the effects of DPI and cromakalim are reversible and that normal proliferation resumes within 24 h of removal of the inhibitors. Alternative mechanisms to account for the stimulation of proliferation with ischemia are unlikely. The similarity in TUNEL staining between control and ischemic cells with positive staining in <2.5% of the cell population excludes a role for cellular apoptosis in the initiation of ischemia-mediated cellular proliferation. Because shear inhibits proliferation, ischemia could represent a return to the presheared state. However, inhibition of ischemia-mediated proliferation by ROS inhibitors or scavengers that do not affect proliferation of statically cultured cells would be evidence against this possibility.

In contrast to ECs from wild-type mice, the Kir6.2−/− and gp91phox−/− ECs showed markedly diminished proliferation in response to flow cessation. As with the chemical inhibitors DPI and cromakalim, the diminished response lasted for ~24 h, after which time normal proliferation resumed. All four models of nonproliferation (2 chemical and 2 knockout) share the characteristic of decreased ROS production with flow cessation compared with wild-type ECs in the absence of inhibitors. Thus the results suggest that ROS provide the signal for initiation of cellular proliferation with flow cessation in flow-adapted ECs. Catalase markedly inhibited the effect of ischemia, while SOD had little effect, suggesting that the proliferative response was initiated by H2O2. The mechanism responsible for the resumption of normal cellular proliferation after 24 h in static culture is not known, but presumably ROS are not involved at that stage.

The presence of ROS has been shown in other studies to promote EC proliferation. Treatment of rat coronary microvascular ECs with pyrogallol or bovine aortic endothelial cells with glucose/glucose oxidase, both of which are ROS generators, resulted in increased cell number and increased DNA synthesis (7, 17). Inhibitors of NADPH oxidase expression also inhibited the proliferation of human ECs from coronary artery, umbilical vein, and dermal microvasculature (1). Similar results were observed in association with antisense-mediated decrease of p22phox in rat coronary microvascular endothelium (7). Treatment of human umbilical vein endothelial cells with VEGF or lysophosphatidylcholine resulted in ROS generation and proliferation that was inhibited by antagonists of NADPH oxidase, including DPI, overexpression of a Rac-1 dominant negative, and transfection with antisense to gp91phox or p22phox (20, 26).

Knockout of gp91phox or treatment with DPI showed complete inhibition of ROS generation and cell proliferation, while only a partial decrease was observed in KATP−/− cells. Although we did not measure it, it seems likely that the membrane potential in Kir6.2-null cells is maintained by other channels that respond only partially to the loss of shear. On the other hand, the results with gp91phox−/− cells indicate that NADPH oxidase is solely responsible for ROS production and that this function is uncompensated in the null cells.

In summary, using an in vitro model of oxygenated ischemia and gene-targeted ECs, we have shown that flow-adapted ECs respond to cessation of flow with cellular proliferation. Proliferation in this model appears to be triggered in response to ROS production. We postulate that ROS promote cell proliferation associated with flow cessation through a signaling cascade initiated by membrane depolarization mediated by KATP channel closure. This response to altered shear stress may represent an effort at neovascularization that is compensatory for loss of shear.

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